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(54) Title: EVOLUTION OF WHOLE CELLS AND ORGANISMS BY RECURSIVE SEQUENCE RECOMBINATION

(57) Abstract

The invention provides methods employing iterative cycles of recombination and selection/screening for evolution of whole cells and organisms toward acquisition of desired properties. Examples of such properties include enhanced recombinogenicity, genome copy number, and capacity for expression and/or secretion of proteins and secondary metabolites.

WHAT IS CLAIMED IS:

1. A method of producing a library of diverse multicellular organsims, the method comprising:

providing a pool of male gametes and a pool of female gametes, wherein at least one of the male pool or the female pool comprises a plurality of different gametes derived from different strains of a species or different species, wherein the male gametes fertilize the female gametes;

permitting at least a portion of the resulting fertilized gametes to grow into reproductively viable organisms;

repeatedly crossing the reproductively viable organisms to produce a library of diverse organisms; and,

selecting the library for a desired trait or property.

2. The method of claim 1, wherein the library of diverse organisms comprise a plurality of plants.

3. The method of claim 2, wherein the plants are selected from: *Gramineae, Fetucoideae, Poacoideae, Agrostis, Phleum, Dactylis, Sorgum, Setaria, Zea, Oryza, Triticum, Secale, Avena, Hordeum, Saccharum, Poa, Festuca, Stenotaphrum, Cynodon, Coix, Olyreae, Phareae, Compositae,* and *Leguminosae*.

4. The method of claim 2, wherein the plants are selected from corn), rice, wheat, rye, oats, barley, pea, beans, lentil, peanut, yam bean, cowpeas, velvet beans, soybean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, sweetpea, sorghum, millet, sunflower, and canola.

5. The method of claim 1, wherein the library of diverse organisms comprise a plurality of animals.

6. The method of claim 5, wherein the animals are selected from non-human mammals and fish.

7. The library produced by the method of claim 1.

8. The method of claim 1, further comprising:

crossing a plurality of selected library members by pooling gametes from the selected members and repeatedly crossing any resulting additional reproductively viable organisms to produce a second library of diverse organisms; and,

selecting the second library for a desired trait or property.

9. The second library made by the method of claim 8.

10. A method of evolving a cell to acquire a desired property, comprising:

(i.) forming protoplasts of a population of different cells;

(ii.) fusing the protoplasts to form hybrid protoplasts, in which genomes from the protoplasts recombine to form hybrid genomes;

(iii.) incubating the hybrid protoplasts under conditions promoting regeneration of cells, thereby producing regenerated cells;

(iv.) repeatedly forming protoplasts from the regenerated cells, fusing the protoplasts to form hybrid protoplasts, in which genomes from the protoplasts recombine to form additional hybrid genomes; incubating the additional hybrid protoplasts under conditions promoting regeneration of cells, thereby producing additional regenerated cells; and,

(v.) selecting or screening to isolate regenerated cells or additionally regenerated cells that have evolved toward acquisition of the desired property.

11. The method of claim 10, wherein the desired property is selected from: heat tolerance, ethanol production, ethanol tolerance, acid, improved production and maintanance of enzyme cofactors, improved production and maintanance of NAD(P)H, and improved glucose transport.

12. The method of claim 10, further comprising repeating steps (i.)-(v.) with regenerated cells in step (iii.) or additional regenerated cells in step (iv.) being used to form the protoplasts in step (i.) until the regenerated cells have acquired the desired property.

13. The method of claim 10, comprising step (iv), wherein step (iv). is performed prior to step (v.).

14. The method of claim 10, wherein the hybrid protoplasts comprise cells having more than two parental genomes.

15. The method of claim 10, wherein the different cells are fungal cells, and the regenerated cells are fungi mycelia.

16. The method of claim 15, wherein protoplasts are provided by treating mycelia or spores with an enzyme.

17. The method of claim 15, wherein the fungal cells are from a fragile strain, lacking capacity for intact cell wall synthesis, whereby protoplast form spontaneously.

18. The method of claim 15, further comprising treating the mycelia with an inhibitor of cell wall formation to generate protoplasts.

19. The method of claim 10, further comprising selecting or screening to isolate regenerated cells with hybrid genomes free from cells with parental genomes.

20. The method of claim 10, wherein a first subpopulation of cells contain a first marker and the second subpopulation of cells contain a second marker, and the method further comprising selecting or screening to identify regenerated cells expressing both the first and second marker.

21. The method of claim 10, wherein the first marker is a membrane marker and the second marker is a genetic marker.

22. The method of claim 10, wherein the first marker is a first subunit of a heteromeric enzyme and the second marker is a second subunit of the heteromeric enzyme.

23. The method of claim 10, further comprising transforming protoplasts with a library of DNA fragments in at least one cycle.

24. The method of claim 23, wherein the DNA fragments are accompanied by a restriction enzyme.

25. The method of claim 10, further comprising exposing the protoplasts to ultraviolet irradiation in at least one cycle.

26. The method of claim 10, wherein the desired property is the expression of a protein, primary metabolite, or secondary metabolite.

27. The method of claim 10, wherein the desired property is the secretion of a protein or secondary metabolite.

28. The method of claim 27, wherein the secondary metabolite is selected from taxol, cyclosporin A, and erythromycin.

29. The method of claim 10, wherein the desired property is capacity for meiosis.

30. The method of claim 10, wherein the desired property is compatibility to form a heterokaryon with another strain.

31. The method of claim 10, further comprising exposing the protoplasts or mycelia to a mutagenic agent in at least one cycle.

32. A method for whole genome shuffling through organized heteroduplex shuffling, the method comprising:

(a). providing chromosomal DNA of an organism which is targeted for shuffling, digesting the chromosomal DNA with one or more restriction enzymes, ligating the chromosomal DNA into a cosmid, the cosmid comprising at least two rare restriction enzyme recognition sites, aliquoting, purifying, and storing sufficient cosmids to represent a complete chromosome;

(b). mutagenizing aliquots of the library in vitro using a mutagen;

(c). transfecting a sample from a plurality of the mutagenized aliquots into a population of target cells;

(d). assaying resulting transfectants for phenotypic improvements;

(e). growing transfected cells harboring a mutant library of the identified cosmid(s) on media and screening the resulting cell colonies for independent mutants conferring an desired phenotype;

(f). isolating and pooling DNA from cells identified in the screening;

(g). dividing the selected pools and digesting at least one sample with a rare-cutting restriction enzyme, pooling the cleaved samples, denaturing the samples, reannealing the samples and religating the samples; and,

(h). transfecting target cells with the resulting heteroduplexes and propagating the cells to allow recombination to occur between the strands of the heteroduplexes in vivo.

33. The method of claim 32, further comprising additionally screening the transfectants.

34. The method of claim 32, further comprising further shuffling the heteroduplexes by recursive in vitro heteroduplex formation and in vivo recombination prior to additionally screening the transfectants.

35. The method of claim 33, further comprising performing an additional mutagenesis step to increase diversity during the shuffling process.

36. The method of claim 32, further comprising combining one or more heteroduplexes into a host chromosome by chromosome integration.

37. The method of claim 36, further comprising repeating steps (a).-(h)., using the organism resulting from chromosome integration as the source for chromosomal DNA in step (a).

38. The method of claim 32, wherein the cosmid comprises restriction sites for Sfr or NotI.

39. The method of claim 32, wherein the transfectants are assayed as a pool from each mutagenized aliquot.

40. The method of claim 32, wherein a positive assay result indicates that a cosmid from a particular aliquot can confer phenotypic improvements and contains large genomic fragments that are suitable targets for heteroduplex mediated shuffling.

41. The method of claim 32, wherein the mutagen is a chemical mutagen.

42. The method of claim 32, wherein growing transfected cells harboring a mutant library of the identified cosmid(s) on media comprises plating the transfected cells on solid media.